The Human Inflammasomes

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ABSTRACT

Two decades of inflammasome research has led to a vast body of knowledge on the complex regulatory mechanisms and pathological roles of canonical and non-canonical inflammasome activation in a plethora of research models of primarily rodent origin. More recently, the field has made notable progress in characterizing human-specific inflammasomes and their regulation mechanisms, including an expansion of inflammasome biology to adaptive immune cells. These exciting developments in basic research have been accompanied by potentially transformative results from large clinical trials and translational efforts to develop inflammasome-targeted small molecule inhibitors for therapeutic use. Here, we will discuss recent findings in the field with a specific emphasis on activation mechanisms of human inflammasomes and their potential role in auto-inflammatory, metabolic and neoplastic diseases.

INTRODUCTION

The human immune system plays a vital role in protecting us against pathogenic microorganisms, harmful substances and cell changes that could result in illness. Myeloid cells and epithelial cells at mucosal surfaces are critical constituents of the innate immune system and play a central role in recognizing imminent danger and mounting a protective response against it. These cells - like many other inflammatory cells - are equipped with a host of plasma membrane-bound and intracellular germline-encoded pattern recognition receptors (PRRs) that detect "pathogen-associated molecular patterns" (PAMPs) and environmental or host-derived stress signals termed "damage-associated molecular patterns" (DAMPs) (Medzhitov, 2008).

A key node of PRR signaling culminates in activation of inflammatory caspases, a subset of evolutionary conserved cysteine-dependent endoproteases that cleave their substrates after specific aspartic acid residues to induce inflammation (Lamkanfi et al., 2002; Van Opdenbosch and Lamkanfi, 2019). Caspase-1 is the prototypical inflammatory caspase grace to its central role in maturing pro-interleukin (IL)-1β and pro-IL-18 into the respective bio-active, secreted cytokines IL-1β and IL-18 (**Figure 1**). Concomitantly, caspase-1 cleaves the cytosol protein gasdermin D (GSDMD) in the central linker region, thus separating the amino-terminal GSDMD domain ($GSDMD_N$) from its carboxy-terminal regulatory domain. This cleavage event allows $GSDMD_N$ to translocate and assemble large homo-oligomeric pores in the plasma membrane through which IL-1ß, IL-18, the related cytokine IL-1α and small, soluble DAMPs exit inflammatory cells and bind to their cognate receptors on effector cells to cause inflammation (Lamkanfi, 2011; Van Opdenbosch and Lamkanfi, 2019). GSDMD pores dissipate ionic gradients across cellular membranes and result in pyroptosis, a lytic cell death mode of inflammatory cells in which plasma membrane rupture and extracellular release of larger DAMPs such as High Mobility Group Box 1 (HMGB1) and lactate dehydrogenase (LDH) occurs through an undefined mechanism involving oligomerization of nerve injury-induced protein 1 (NINJ1) (Luchetti et al., 2021). Caspase-1 is not unique in its ability to induce pyroptosis. Caspase-11 in rodents and its human ortholog caspases 4 and 5 can also cleave GSDMD to induce pyroptosis, although caspase-1 is still required under these conditions for ensuring that proIL-1β and proIL-18 are cleaved into the respective secreted cytokines (Kayagaki et al., 2015; Schmid-Burgk et al., 2015; Shi et al., 2015).

Activation of inflammatory caspases must be tightly controlled to avoid the potentially destructive impact of excessive or unwarranted cell death and inflammatory tissue damage. The mechanism of proximity-induced auto-activation is a key regulatory checkpoint in this respect, ensuring that caspases 1, 4, 5 and 11 only gain proteolytic activity when their zymogens reach high local concentrations upon their recruitment in large multi-protein complexes. Canonical inflammasomes are multi-protein complexes that are assembled in the cytosol of myeloid and epithelial cells to engage caspase-1,

whereas caspases 4, 5 and 11 are activated in the so-called 'non-canonical inflammasome pathway' (Lamkanfi and Dixit, 2014; Van Opdenbosch and Lamkanfi, 2019). Two decades of inflammasome research has led to a vast body of knowledge on the intricate molecular mechanisms and (patho)physiological roles of canonical and non-canonical inflammasome activation in a plethora of research models of primarily rodent origin, as has been extensively discussed (Lamkanfi and Dixit, 2014; Van Opdenbosch and Lamkanfi, 2019; Voet et al., 2019). More recently, the field has made great progress in characterizing human-specific inflammasomes and regulation mechanisms, and inflammasome biology has expanded to adaptive immune cells. These exciting developments are paralleled by the publication of potentially transformative results from large clinical trials and efforts to develop inflammasome-targeted inhibitors for therapeutic use (Chauhan et al., 2020; Van Gorp et al., 2019). Here, we will discuss recent findings with a specific emphasis on human inflammasomes and their roles in auto-inflammatory and metabolic diseases, and suggest potential directions for future research.

HUMAN INFLAMMASOMES AND THEIR ACTIVATION MECHANISMS

The assembly of the canonical inflammasomes in the cytosol is induced when signs of imminent threat is detected by a specific subset of PRRs. Detection of PAMPs and DAMPs by such PRRs triggers oligomerization of these inflammasome sensor proteins and leads to recruitment of the effector protease caspase-1 via the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC)(Van Opdenbosch and Lamkanfi, 2019). The PRRs Absent in melanoma 2 (AIM2), Caspase Recruitment Domain 8 (CARD8), Pyrin and the "nucleotide-binding oligomerization domain and leucine-rich repeat-containing receptor" (NLR) family members NLRP1, NLRP3 and NLRC4 have all been confirmed to assemble canonical inflammasomes that recruit and activate caspase-1 in humans, and several additional complexes have been proposed (**Figure 2**). Below, we will discuss the activation mechanisms of these different human inflammasomes and their roles in disease.

THE CANONICAL INFLAMMASOMES

The human NLRP1 inflammasome

The NLRP1 inflammasome was the first inflammasome described in 2002 (Martinon et al., 2002). However, it's not until recently that we have started to understand the molecular mechanisms controlling NLRP1 activation. In addition to the central NACHT domain and Leucine-rich repeat (LRR) motifs also found in other NLR family members, human NLRP1 is extended in its carboxy-terminus with a function-to-find (FIIND) domain followed by a CARD domain (**Figure 2**). The FIIND domain is unique to NLRP1 and the related inflammasome sensor CARD8 (discussed below), and is further subdivided

into the ZU5 and UPA subdomains. About half of the cellular NLRP1 pool undergoes autocleavage in its FIIND domain. However, the proteolytically separated ZU5 and UPA subdomains remain non-covalently associated in resting conditions (D'Osualdo et al., 2011; Finger et al., 2012) (**Figure 4**). Recent structural and biochemical insights clarified how binding of the homologous the cytosolic dipeptidyl peptidases (DPP)8 and DPP9 to the FIIND domain represses NLRP1 activation (Hollingsworth et al., 2021; Huang et al., 2021). These studies showed that DPP9 interacts with both full-length NLRP1 as well as with the carboxy-terminal fragment of autocleaved NLRP1, thus providing a molecular mechanism explaining how DPP8/DPP9 inhibitors such as the boronic dipeptide Val-boroPro (VbP) de-repress NLRP1 for activation (Hollingsworth et al., 2021; Huang et al., 2021). More specifically, DPP8/DPP9 inhibitors cause the autoinhibitory amino-terminal polypeptide chain of NLRP1 to be degraded by the 26S proteasome, thereby licensing the carboxy-terminal UPA-CARD fragment of NLRP1 to scaffold a functional inflammasome that drives caspase-1 activation (Ball et al., 2020; Finger et al., 2012; Martinon et al., 2002; Zhong et al., 2016).

VbP and other DPP8/DPP9 inhibitors also activate the NLRP1b inflammasome in rodent macrophages (Chui et al., 2019; de Vasconcelos et al., 2019; Okondo et al., 2017). However, notable differences exist between human NLRP1 and its rodent orthologs. Firstly, humans have only one NLRP1 gene, whereas murine chromosome 11 encodes two functional NLRP1 genes - namely *Nlrp1a* and *Nlrp1b* - in addition to the predicted pseudogene *Nlrp1c* (Boyden and Dietrich, 2006). Second, the amino-terminal regions of murine and human NLRP1 differ significantly, including human NLRP1 containing an auto-inhibitory amino-terminal PYD domain that is missing in murine NLRP1a and NLRP1b. Third, human NLRP1 requires ASC (Ball et al., 2020; Finger et al., 2012; Martinon et al., 2002; Zhong et al., 2016), whereas the latter is dispensable for caspase-1 activation by murine NLRP1a and NLRP1b (Masters et al., 2012; Van Opdenbosch et al., 2014). Fourth, unlike murine keratinocytes, human keratinocytes express high NLRP1 levels (Sand et al., 2018). Fifth, the NLRP1b allele of certain murine inbred strains responds to *Bacillus anthracis* lethal toxin (LeTx), contrary to human NLRP1 that resists activation by LeTx (Boyden and Dietrich, 2006). Moreover, a recent report showed that long double-stranded RNA (dsRNA) binding at the LRR motifs activates human NLRP1 in the cytosol of virus-infected keratinocytes, whereas murine NLRP1 orthologs are unresponsive to cytosolic dsRNA (Bauernfried et al., 2021). Finally, the NLRP1 inflammasome is required for IL-1β and IL-18 secretion in UVB-irradiated human epidermal keratinocytes, whereas UVB-induced IL-1β secretion in murine skin is primarily mediated by infiltrating phagocytes (Fenini et al., 2018; Sand et al., 2018). Human NLRP1 also senses viral infections of the airway epithelium by exploiting the unstructured linker region between the PYD and NACHT domains in its amino-terminal region as a decoy substrate that is destabilized and degraded by the proteasome when it gets cleaved by promiscuous viral proteases such as 3CL proteases of coronaviruses or the 3C protease of rhinoviruses that cause the common cold (Planes et al., 2022; Robinson et al., 2020; Tsu et

al., 2021). Although cleavage by viral proteases is specific to human NLRP1, the mechanism is reminiscent of that used by murine NLRP1b to detect LeTx in murine macrophages (Chavarria-Smith et al., 2016; Chavarria-Smith and Vance, 2013; Levinsohn et al., 2012). Collectively, these recent findings have uncovered significant functional differentiation between human and rodent NLRP1, while their molecular activation mechanisms may seem evolutionarily better conserved.

The human CARD8 inflammasome

CARD8 is an inflammasome sensor that is unique to humans and other primates. CARD and NLRP1 have in common that they are the only human proteins containing a FIIND domain, which lays adjacent to the CARD in their carboxy-terminus (**Figure 2**). Also analogous to NLRP1, the CARD8 FIIND domain undergoes autoproteolytic cleavage in the short linker separating its ZU5 and UPA subdomains, which remain non-covalently associated in the DPP8/DPP9-suppressed conformation (D'Osualdo et al., 2011; Johnson et al., 2018). However, CARD8 lacks the central NACHT domain and LRR motifs, as well as the amino-terminal PYD domain of human NLRP1. Instead, CARD8 contains an unstructured region in the amino-terminal polypeptide sequence that precedes its FIIND domain. As such, CARD8 may be regarded as a truncated paralog of human NLRP1. However, ASC is strictly required for assembly of the human NLRP1 inflammasome, whereas the CARD8 CARD is incapable of interacting with ASC and recruits caspase-1 directly (Ball et al., 2020) (**Figure 4**).

Intriguingly, whereas VbP activates the NLRP1 inflammasome in human keratinocytes and lung epithelial cells, it induces CARD8 inflammasome activation in primary human monocytes, diverse immortalized myeloid leukemia cell lines such as the widely used THP-1 cells, as well as in resting $CD4^+$ and $CD8^+$ T lymphocytes (Johnson et al., 2020; Johnson et al., 2018; Linder et al., 2020). Consistent with pro-IL-1ß being primarily expressed in myeloid cells, VbP-induced CARD8 inflammasome activation triggered pyroptosis of resting T lymphocytes without eliciting IL-1ß secretion (Johnson et al., 2020; Linder et al., 2020). Activated human T cells completely resist CARD8-mediated pyroptosis despite expressing detectable levels of CARD8, caspase-1 and GSDMD (Johnson et al., 2020; Linder et al., 2020). This intriguing observation warrants further investigation and might reveal novel T cell receptor signaling nodes that actively suppress CARD8 inflammasome activation in activated T cells. Collectively, these findings have firmly established CARD8 as an inflammasome sensor in humans and extended canonical inflammasome signaling from innate immune cells to T lymphocytes.

A recent study showed a role for the CARD8 inflammasome in detecting human immunodeficiency virus-1 (HIV-1) infection in macrophages and quiescent $CD4^+$ cells following treatment with HIV-specific non-nucleoside reverse transcriptase inhibitors (Wang et al., 2021). HIV-1 is a T-cell tropic infection that is effectively suppressed by modern antiretroviral therapy apart from latent viral reservoirs that persist primarily in quiescent $CD4^+$ T cells and possibly in macrophages. The CARD8 inflammasome is

engaged in these cells when antiretroviral drugs targeting HIV-1 Pol cause dimerization and premature activation of HIV-1 protease. This, in turn, results in HIV-1 protease-directed truncation and proteasome-dependent degradation of the auto-inhibitory amino-terminal CARD8 polypeptide chain, which releases the carboxy-terminal UPA-CARD fragment of CARD8 to recruit caspase-1 and induce pyroptosis (Wang et al., 2021). As discussed earlier, this biochemical activation mechanism is reminiscent of that employed by the NLRP1 inflammasome to detect enteroviral proteases in keratinocytes and bronchial epithelial cells. It will be of major interest to explore whether this mechanism may contribute to clinical HIV cure strategies by helping to reduce seeding of latent HIV-1 reservoirs and eliminating residual HIV-1-infected cells in patients, although this may as well impose selective pressure on HIV-1 to evade this sensing mechanism or evolve alternative counterstrategies. In parallel to further addressing the role of CARD8 in T cell tropic infections, future studies should examine whether CARD8-induced T cell pyroptosis has a physiological role in regulating the homeostatic processes of positive and negative selection of the T cell repertoire.

The human NLRP3 inflammasome

The NLRP3 inflammasome is by far the most studied inflammasome. This is rooted in its responding to a wide range of stimuli of endogenous, environmental and microbial origin, and in its anticipated role in driving chronic inflammatory pathology in patients with cancer, metabolic, auto-inflammatory, autoimmune and neurodegenerative diseases (Chauhan et al., 2020; Van Gorp et al., 2019; Van Opdenbosch and Lamkanfi, 2019; Voet et al., 2019).

A vast body of research, conducted primarily in murine macrophages, has led to the now entrenched concept that NLRP3 activation proceeds through a bi-phasic process involving sequential priming and activation steps (Lamkanfi and Dixit, 2014). The priming signal usually is delivered by inflammatory cytokine receptors, membrane-bound Toll-like receptor (TLR) and intracellular PRRs that share the ability to induce NF-κB-mediated transcriptional upregulation of NLRP3 expression levels concomitant with induction of pro-IL-1ß gene expression (Bauernfeind et al., 2009) (**Figure 3**). Experimental conditions in which NLRP3 undergoes non-transcriptional priming have also been reported. Under these conditions, a short “pulse” with agonists of the above-mentioned PRRs that is considered too brief to induce NF-κB-mediated transcriptional upregulation of inflammasome components licenses NLRP3 for subsequent activation (Fernandes-Alnemri et al., 2013; Gritsenko et al., 2020a; Humphries et al., 2018; Juliana et al., 2012; Lin et al., 2014; Song et al., 2017; Xing et al., 2017). Changes in NLRP3 post-translational modifications (PTMs) such as phosphorylation and ubiquitination status are thought to underlie non-transcriptional priming of NLRP3, although the precise mechanisms by which these PTMs ready NLRP3 for activation are incompletely understood.

Priming clearly is a prerequisite for NLRP3 inflammasome activation in rodent macrophages and in human monocyte-derived macrophages (Bauernfeind et al., 2009; Gritsenko et al., 2020b). However, canonical NLRP3 activation also occurs in unprimed primary human monocytes and in unprimed and undifferentiated conditions in the human leukemic cell line THP-1 (Gritsenko et al., 2020b; O'Brien et al., 2017). This pathway features ASC speck formation, caspase-1-mediated pyroptosis and GSDMD-dependent secretion of constitutively expressed IL-18 in the absence of detectable IL-1ß secretion. This suggests either that priming might not be an absolute requirement for NLRP3 activation under specific conditions, or that NLRP3 might exist in a 'pre-primed' state in these cells.

Lipopolysaccharide (LPS) from Gram-negative bacteria is the most commonly used agent to prime rodent macrophages and human monocyte-derived macrophages for subsequent 'signal 2' agents such as extracellular ATP, bacterial toxins and particulate substances such as monosodium urate (MSU) crystals and ß-amyloid (Lamkanfi and Dixit, 2014). Many of these 'signal 2' activators promote a drop in the cytosolic K^+ concentrations that are thought to converge on NLRP3 activation by instigating activating conformational changes in NLRP3 (Munoz-Planillo et al., 2013). Intriguingly, however, LPS alone activates the NLRP3 inflammasome in primary human blood monocytes and phorbol ester-differentiated THP-1 cells without necessitating such classical 'signal 2' agents (Netea et al., 2009; O'Brien et al., 2017). LPS-induced NLRP3 activation was recently also shown to trigger pyroptosis in differentiated THP-1 cells (O'Brien et al., 2017). In contrast, TLR4 stimulation by LPS alone activates an 'alternative' NLRP3 pathway that promotes IL-1ß secretion without eliciting pyroptosis or assembly of ASC specks in BLaER1 cells, a transdifferentiated human monocyte/macrophage cell line (Gaidt et al., 2016). Undoubtedly, a better understanding of the molecular basis of 'one-step' NLRP3 activation will shed further light on fundamental aspects of NLRP3 inflammasome activation.

Recent studies have uncovered a remarkable cell type-specific redundancy in detection of bacterial PAMPs and effector proteins by human inflammasome sensors. This is illustrated by *Clostridium difficile* Toxin A and B (TcdA/B) activating the NLRP3 inflammasome in human monocyte-derived macrophages, whereas instead these bacterial exotoxins activate the Pyrin inflammasome in human blood monocytes and murine macrophages (Mangan et al., 2021; Van Gorp et al., 2016; Xu et al., 2014). This is likely due to Pyrin expression levels being too low to support inflammasome responses in unprimed monocyte-derived macrophages as ascertained by prolonged priming with LPS or type I or II interferons (IFN) rescuing Pyrin inflammasome activation in these cells (Mangan et al., 2021). Moreover, the NLRP3 inflammasome in human mononuclear cells and in murine macrophages indirectly senses activation of small Rho GTPases by bacterial pathogens and toxins such as Rac1-activating *Chlamydophila pneumoniae* infection and the Rac2-activating CNF1 toxin encoded by *Escherichia coli* (Dufies et al., 2021; Eitel et al., 2012). Furthermore, Pam_3CSK_4-primed primary human monocytes – as do murine macrophages - activate the NLRP3 inflammasome following cytosolic

delivery of dsRNA (Bauernfried et al., 2021; Kanneganti et al., 2006). As discussed earlier, this contrasts markedly with the situation in human keratinocytes and bronchial epithelial cells that respond with NLRP1 inflammasome activation (Bauernfried et al., 2021). Finally, cytosolic dsDNA detection by the cGAS-STING signaling axis leads to downstream activation of the canonical NLRP3 inflammasome in human monocytes, whereas the AIM2 inflammasome is tasked with direct sensing of microbial and host-derived dsDNA in the cytosol of murine macrophages (Gaidt et al., 2017).

Important progress has recently also been made in unravelling the structural underpinnings of NLRP3 activation. A notable discovery in this regard is that auto-inhibited full-length human NLRP3 exists as a pre-assembled ring of five NLRP3 dimers in which the central NACHT domains are aligned along the apical axis and the PYD domains are largely shielded inside the spherical structure as to preclude inadvertent recruitment of ASC (Hochheiser et al., 2021). A similar structure that consists of six to eight dimers has also been reported for murine NLRP3 (Andreeva et al., 2021). These cryo-electron microscopy structures will prove invaluable for formulating and testing molecular models of NLRP3 activation, and are likely to advance structure-aided development of therapeutic NLRP3 inhibitors. Indeed, the small molecule NLRP3 inhibitor CRID3 (also named MCC950) targets the central NACHT domain of NLRP3 (Coll et al., 2019; Vande Walle et al., 2019). Recently reported X-ray and cryo-electron microscopy structures now mapped the molecular binding pocket of CRID3 to a cleft at the interface of the four subdomains of the NACHT in the inactive, ADP-bound conformation of NLRP3 (Dekker et al., 2021; Hochheiser et al., 2021). CRID3 binding in this hydrophobic pocket stabilizes the inactive conformation of NLRP3. Notably, molecular modelling of the active, open conformation of NLRP3 suggests that major spatial rearrangements in the NACHT cause the entire inhibitor binding pocket to disappear, thus providing molecular insight as to why disease-associated activating mutations in and around the central NACHT region may render CRID3 less effective in blocking autoinflammatory disease-associated NLRP3 mutants (Dekker et al., 2021; Hochheiser et al., 2021; Vande Walle et al., 2019).

Structural analysis of how NLRP3 interacts with NEK7 has also been obtained recently (Sharif et al., 2019). The structure revealed that this mitotic serine/threonine kinase acts as a co-factor that physically bridges the space between adjacent NLRP3 subunits in the oligomeric NLRP3 structure, explaining how NEK7 binding promotes NLRP3 inflammasome activation independently of its kinase activity (He et al., 2016; Schmid-Burgk et al., 2016; Shi et al., 2016). Notably, NLRP3 attains only a semi-open conformation when NEK7 is recruited, suggesting that it represents an intermediate step in how NLRP3 transitions from a closed, inactive into a fully open, active conformation that can engage ASC and assemble the NLRP3 inflammasome (Hochheiser et al., 2021; Sharif et al., 2019). Intriguingly, a recent report suggested that the requirement for NEK7 in licensing NLRP3 inflammasome activation

may be bypassed by the kinase TAK1 under certain conditions in murine macrophages and human myeloid cell lines (Schmacke et al., 2019).

The human NAIP/NLRC4 inflammasome

Whereas many questions about the NLRP3 inflammasome remain to be addressed, the activation mechanisms of the NLRC4 inflammasome are relatively well-understood. NLRC4 is unique amongst the different inflammasome-promoting NLRs in that NLRC4 oligomer assembly is seeded by another NLR family member (**Figure 2**). The human NLRC4 inflammasome is activated when human NLR apoptosis inhibitory protein (NAIP) senses bacterial flagellin or components of bacterial type III secretion systems (T3SS) in the cytosol of infected human monocyte-derived macrophages (Kortmann et al., 2015; Reyes Ruiz et al., 2017). The NAIP splice variant most prominently expressed in immortalized human monocytic cell lines appears to more selectively sense the T3SS needle protein (Yang et al., 2013) (**Figure 4**). Whereas human NAIP has evolved to recognize multiple bacterial ligands, the murine genome encodes several functional NAIP proteins that each sense distinct bacterial ligands. Mouse NAIP1 activates the NLRC4 inflammasome when it detects bacterial T3SS needle proteins, NAIP2 senses the T3SS inner rod protein, and NAIP5 and NAIP6 recognize flagellin in the cytosol of murine macrophages (Duncan and Canna, 2018; Lamkanfi and Dixit, 2014). Expression of murine NAIP genes is controlled by interferon regulatory factor 8 (IRF8) (Karki et al., 2018). Whether this also holds for human NAIP remains to be examined. The ligand-bound NAIP proteins undergo conformational changes that promote molecular interactions between the NACHT domains of NAIP and NLRC4, hence nucleating a large NLRC4 oligomeric platform that recruits and engages caspase-1 either directly or indirectly through ASC (Duncan and Canna, 2018). As will be discussed further, the notable expression of NAIP/NLRC4 inflammasome in epithelial cells lining the gastro-intestinal tract and in the myeloid compartment may explain why gain-of-function mutations in human NLRC4 cause autoinflammatory syndromes associated with pathogenic enterocolitis together with exceptionally high levels of circulating IL-18 (Duncan and Canna, 2018; Van Gorp et al., 2019).

The human AIM2 inflammasome

Being a non-NLR inflammasome sensor, the domain architecture of Absent In Melanoma 2 (AIM2) differs from that of the NLRs discussed above. AIM2 contains a PYD domain in its amino-terminal through which it interacts with ASC and a carboxy-terminal hematopoietic IFN-inducible nuclear protein (HIN) domain that can bind dsDNA from microbial and parasitic origin (**Figure 2**). Activation of the AIM2 inflammasome when cytosolic dsDNA is bound to its HIN domain has been extensively studied in murine and human macrophages, and the myeloid leukemia cell line THP-1 (Kumari et al.,

2020) (**Figure 4**). Intriguingly, however, in human primary monocytes cytosolic dsDNA instead activates the NLRP3 inflammasome (Gaidt et al., 2017). These observations suggest that NLRP3 inflammasome activation by dsDNA may act as a backup mechanism that is triggered when AIM2 activation fails, such as in cell types that express AIM2 below a sufficiently high threshold level. AIM2 expression levels can be upregulated by type I IFN. Type I IFNs are cytokines that also induce expression of guanylate-binding proteins (GBPs), an evolutionarily conserved family of abundantly expressed interferon-inducible GTPases with diverse functions in anti-microbial host defense (Kim et al., 2016). One such role of GBPs - demonstrated in *Toxoplasma gondii*-infected human monocyte-derived macrophages and in the human leukemia cell line THP-1 - is to induce lysis of the parasite's plasma membrane and the pathogen-containing vacuole, thus exposing the parasite's genomic DNA to AIM2 sensing and promoting ASC- and caspase-8-dependent apoptosis of infected cells (Fisch et al., 2019). Moreover, AIM2 was suggested to interact with the innate immune sensors ZBP1 and pyrin in PMA-differentiated THP-1 cells following infection with Herpes simplex virus 1 (Lee et al., 2021). Aside from sensing microbial DNA as highlighted above, there is evidence suggesting a role for AIM2 in recognizing self-DNA in the context of neoplasms, and autoinflammatory and autoimmune diseases (Kumari et al., 2020).

The human Pyrin inflammasome

Pyrin (also named TRIM20) is an inflammasome-assembling member of the tripartite motif-containing (TRIM) family of PRRs. Pyrin is primarily expressed in human monocytes and neutrophils. The Pyrin inflammasome responds indirectly to microbial toxins that covalently modify and inhibit RhoA GTPase activity such as RhoA glucosylation by the *Clostridium difficile* Toxins A and B (TcdA/B) (Gao et al., 2016; Van Gorp et al., 2016; Xu et al., 2014). Pyrin contains a PYD domain in its amino-terminus through which it interacts with ASC, followed by a B-box and coiled-coil regions in its centre and a carboxy-terminal PRY/SPRY domain (**Figure 2**). Notably, the carboxy-terminal PRY/SPRY domain (also called B30.2 domain) is dispensable for inflammasome activation because murine Pyrin assembles an inflammasome despite lacking the PRY/SPRY domain in its carboxy-terminus. However, the PRY/SPRY domain of human Pyrin is likely to modulate inflammasome responses because uncontrolled inflammasome activation in Familial Mediterranean Fever (FMF) patients are most often due to gain-of-function mutations in this region (Van Gorp et al., 2016; Van Gorp et al., 2019).

Under homeostatic conditions, two phosphorylated serine residues (S208 and S242) in the amino-terminal portion of the protein recruit 14-3-3 proteins that keep Pyrin in an inactive conformation (Masters et al., 2016). Dephosphorylation of these serine residues dissociates Pyrin from 14-3-3 proteins in a first checkpoint leading to Pyrin inflammasome activation (**Figure 4**). The critical importance of this regulatory mechanism is highlighted by an inborn syndrome named Pyrin-

associated autoinflammation with neutrophilic dermatosis (PAAND), in which S242R or E244K mutations in Pyrin reduce 14-3-3 protein binding and cause spontaneous Pyrin inflammasome activation (Masters et al., 2016; Moghaddas et al., 2017).

Microtubules are thought to play an important role in a second checkpoint controlling Pyrin inflammasome activation because colchicine, a microtubule polymerization inhibitor that is clinically used to treat gout and Familial Mediterranean Fever (FMF), inhibits TcdA/TcdB-induced Pyrin inflammasome activation in murine macrophages and primary human monocytes (Gao et al., 2016; Park et al., 2016; Van Gorp et al., 2016). Intriguingly, peripheral blood mononuclear cells (PBMCs) and monocytes isolated from patients with FMF-causing gain-of-function mutations in Pyrin are able to overcome the requirement for microtubules and efficiently activate the Pyrin inflammasome in the presence of colchicine (Van Gorp et al., 2016). This atypical response of FMF PBMCs to TcdA/TcdB intoxication inspired the development of a functional diagnostic assay that identifies FMF patients amongst autoinflammatory disease patients with high sensitivity and specificity, and may help to expedite differential diagnosis of the disease (Van Gorp et al., 2020).

THE NON-CANONICAL INFLAMMASOME PATHWAY

The canonical inflammasomes discussed above all serve to recruit and activate caspase-1. Activation of the human inflammatory caspases 4 and 5 - and their murine ortholog caspase-11 – is controlled by the non-canonical inflammasome pathway (Kayagaki et al., 2011; Schmid-Burgk et al., 2015; Shi et al., 2014; Van Opdenbosch and Lamkanfi, 2019). This pathway is engaged in murine macrophages, human monocytes and immortalized epithelial cell lines upon transfection or electroporation with LPS and in response to infection with Gram-negative bacteria such as enterohemorrhagic *Escherichia coli* (EHEC), *Vibrio cholerae* and *Haemophilus influenzae.* GSDMD is a common substrate of inflammatory caspases and GSDMD cleavage by caspases 4, 5 and 11 autonomously induces pyroptosis (**Figure 3**). The assembly of GSDMD plasma membrane pores also results in K^+ efflux, which consequently promotes NLRP3 inflammasome-dependent maturation of IL-1β and IL-18 (Baker et al., 2015; Kayagaki et al., 2015; Ruhl and Broz, 2015; Schmid-Burgk et al., 2015).

We discussed above the role of guanylate-binding proteins (GBPs) in regulating activation of the AIM2 inflammasome. Notably, GBPs also appear to play an essential role in controlling caspase-4 activation in the non-canonical inflammasome pathway. GBP1 was recently shown to coordinate the release and detection of cytosolic LPS in IFNγ-primed THP-1 and primary monocyte-derived macrophages infected with *Salmonella* Typhimurium, as well as in IFNγ-primed HeLa cells following transfection or electroporation with LPS. GBP1 does so by binding to cytosolic exposed LPS-containing bacterial

membranes and by recruiting GBP2-4 to assemble a multi-protein complex, which on its turn serves to recruit and activate caspase-4 (Fisch et al., 2019; Santos et al., 2020; Wandel et al., 2020).

Attesting to the key role of non-canonical inflammasome in controlling microbial infections, recent studies uncovered two novel evasion strategies by which the Gram-negative pathogen *Shigella flexneri* evades pyroptosis induction by the non-canonical inflammasome to safeguard it replicative niche (Van Hauwermeiren and Lamkanfi, 2021). OspC3 is the name of an important virulence factor expressed by *S. flexneri* that was recently shown to catalyze a novel post-translational modification termed 'ADP riboxanation' that modifies specific arginine residues in both human caspase-4 and murine caspase-11 (Li et al., 2021). Intriguingly, this modification hinders both autocleavage of these inflammatory caspases as well as their cleavage of GSDMD. The virulence factor IpaH7.8 of *S. flexneri* employs yet another creative mechanism to dodge pyroptosis and evade immune recognition. Notably, IpaH7.8 specifically targets human GSDMD - but not its murine ortholog - for ubiquitination and degradation by the host's proteasome machinery (Luchetti et al., 2021). Consistent with humans being significantly more sensitive to shigellosis compared to mice, this discovery appears to have uncovered a remarkable example of co-evolution shaping virulence interactions between bacterial pathogens and their natural hosts.

INFLAMMASOMES IN AUTO-INFLAMMATORY, METABOLIC AND NEOPLASTIC DISEASES

Auto-inflammatory diseases

Inflammasomes are indispensable for protecting us against a broad range of bacterial, viral and protozoal infections. However, uncontrolled and excessive inflammasome activation without apparent cause may also cause harm to the body as highlighted by inflammasome-driven pathologies in various auto-immune and auto-inflammatory diseases. A distinguishing characteristic of auto-inflammatory diseases is that they are primarily driven by aberrant or excessive activation of innate immune cells with dysregulated secretion of inflammatory cytokines, with T and B lymphocytes playing only a marginal role. This contrasts with autoimmune diseases in which adaptive immune cells and autoantibodies are the prime culprits in the disease.

Auto-inflammatory diseases in which inflammasomes are known or suspected to be in the drivers' seat are often referred to as 'inflammasomopathies' (Van Gorp et al., 2019). With the notable exceptions of *CARD8* and *AIM2*, monogenic (inborn) inflammasomopathies caused by activating mutations in the genes encoding each of the inflammasome sensor proteins discussed above have been reported. Gain-of-function mutations in the *NLRP3* gene cause three periodic fever syndromes that collectively known as Cryopyrin-Associated Periodic Syndromes (CAPS). Patients with CAPS are further categorized as having familial cold autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS) and

neonatal-onset multisystem inflammatory disease (NOMID) although patients with an intermediate spectrum of symptoms also exist. Periodically recurring fevers, skin rash, general fatigue and joint pains are common symptoms of CAPS patients, whereas disease severity and the range of affected organs is most extensive in NOMID, and mildest in FCAS patients (Van Gorp et al., 2019). CAPS has an autosomal dominant inheritance and exacerbated IL-1β production due to uncontrolled NLRP3 inflammasome activation has been identified as the main disease mechanism. Consequently, IL-1 blockade with clinically approved biologics is in general highly effective in controlling disease symptoms in CAPS patients.

Mutations in the *MEFV* gene encoding Pyrin cause two auto-inflammatory diseases with a differential clinical presentation. With an estimated 150,000 patients around the world, FMF is considered the most prevalent monogenic autoinflammatory disease in humans (Van Gorp et al., 2019). Notably, genetic deletion of GSDMD was recently shown to fully rescue inflammatory pathology in mouse models of CAPS and FMF, thus pointing to GSDMD-induced pyroptosis as a potential therapeutic target in these inflammatory diseases. Unlike CAPS, however, FMF most commonly is a recessive disease, and disease symptoms of FMF patients are generally well-controlled by colchicine therapy. As mentioned earlier, mutations in the amino-terminal region of Pyrin that alter the 14-3-3 protein binding site (S242R or E244K) cause another syndrome called Pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND) (Masters et al., 2016; Moghaddas et al., 2017). Unlike FMF, PAAND has a autosomal dominant inheritance pattern, and typical PAAND symptoms include sterile skin abscesses, febrile episodes that can last for weeks, myalgia, myositis and fatigue.

Auto-inflammatory patients with gain-of-function mutations in the gene coding for the inflammasome sensor NLRC4 have also been described (Van Gorp et al., 2019). NLRC4 mutations cause a varying spectrum of inflammatory symptoms, ranging from mild urticarial rash in patients suffering from Familial Cold Autoinflammatory Syndrome 4 (FCAS4) to recurrent autoinflammation associated with poor overall growth and severe enterocolitis with an onset in infancy (AIFEC) and increased risk of potentially life-threatening macrophage-activation syndrome (MAS). In contrast to the autoinflammatory syndromes described earlier, IL-1 blocking therapy are only partially effective in treating patients with NLRC4 gain-of-function mutations due to additional contributions of IL-18 and IFNγ in the etiology of NLRC4-dependent auto-inflammation (Van Gorp et al., 2019).

Consistent with NLRP1 being a key inflammasome sensor in keratinocytes, pathogenic germline gain-of-function mutations in the *NLRP1* gene are associated with dyskeratosis and skin cancer susceptibility in patients. NLRP1-driven skin inflammatory diseases have been named MSPC (multiple self-healing palmoplantar carcinoma), FKLC (familial keratosis lichenoides chronica) and NLRP1-associated autoinflammation with arthritis and dyskeratosis (NAIAD), depending on where the disease-causing mutations in NLRP1 are situated (Alehashemi and Goldbach-Mansky, 2020). Notably, a recent report

also described siblings with juvenile-onset recurrent respiratory papillomatosis (JRRP) that is caused by homozygous NLRP1 gain-of-function mutations (Drutman et al., 2019).

In addition to the aforementioned monogenic auto-inflammatory disorders, inflammasomes may also contribute to pathogenesis of more common inflammatory diseases such as ulcerative colitis, Crohn's disease and rheumatoid arthritis. Consistent herewith, the recombinant IL-1 receptor antagonist Anakinra has been approved for the treatment of rheumatoid arthritis (Nikfar et al., 2018). Genetic deletion of NLRP3 was also found to rescue clinical disease parameters in a mouse model of inflammatory arthritis that is driven by myeloid-specific deficiency in the anti-inflammatory protein A20 (Vande Walle et al., 2014). The NLRP3 inflammasome is also suspected to play a central role in crystal-induced arthropathies such as gout, which is caused by deposition of monosodium urate (MSU) crystals in the joints and other tissues (Martinon et al., 2006). Based on positive results from clinical trials (Janssen et al., 2019; So et al., 2010), IL-1 blocking biologics are increasingly being considered for the treatment of gout in patients whose disease is unresponsive to standard therapies. Finally, results from a prospective, open-label study showed that the IL-1 targeted biologic rilonacept reduced symptoms in a small cohort of eight patients suffering from Schnitzler's syndrome, a rare autoinflammatory disorder associated with chronic urticaria, monoclonal IgM or IgG gammopathy and other systemic inflammatory symptoms (Krause et al., 2012).

Metabolic diseases and tumorigenesis

Chronic, low-grade inflammation has long been known to increase risk for metabolic diseases such as atherosclerosis, type 2 diabetes, obesity and non-alcoholic fatty liver disease (Hotamisligil, 2006). Lipids and cholesterol crystals are amongst the DAMPs that trigger activation of the NLRP3 inflammasome. Moreover, macrophages and neutrophils with high expression levels of NLRP3 inflammasome components are abundantly detected in atherosclerotic plaques, which are formed on the inner walls of arterial vessels as a result of the deposition of oxidized cholesterol-containing low-density lipoprotein (LDL) and other lipids (Paramel Varghese et al., 2016). Key results from the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial recently established a prominent role for IL-1β in driving adverse cardiovascular events in patients (Ridker et al., 2017a). This randomized, double-blind multi-center phase 3 trial examined the effect of canakinumab, a therapeutic IL-1β neutralizing antibody, in controlling cardiovascular outcomes in a large cohort of more than 10,000 atherosclerosis patients with systemic low-grade inflammation who previously experienced a myocardial infarction or stroke. The study reached its primary efficacy end point by demonstrating a significantly lower rate of recurrent cardiovascular events in the treatment arms of the study compared to placebo. Importantly, protection from recurrent cardiovascular events in CANTOS was correlated with on-treatment reduction in systemic inflammatory biomarkers and

occurred independently of lipid-level lowering (Ridker et al., 2017a). A recently reported post-hoc analysis of CANTOS revealed that canakinumab also reduced the risk for gout flares by about half compared to placebo without modulating serum uric acid levels (Solomon et al., 2018). As we have extensively discussed (Van Gorp and Lamkanfi, 2019), a significant body of evidence suggests a role for IL-1ß in regulating carcinogenesis. This link has been confirmed by a secondary analysis of CANTOS data showing that IL-1β neutralization with canakinumab reduced lung cancer incidence and lung cancer-associated mortality in a dose-dependent manner (Ridker et al., 2017b). Collectively, these exciting results provide large-scale in-human evidence for the hypothesis that low-grade inflammation contributes to the pathobiology of atherothrombosis, and support the clinical development of NLRP3-targeted drugs for atherosclerosis, gout and possibly cancer. Furthermore, selective NLRP3 inhibition may alleviate the increased risk of non-opportunistic infections that accompanied IL-1 blockade in CANTOS and other clinical studies (Ridker et al., 2017a).

CONCLUSIONS AND FUTURE PERSPECTIVES

Since its inception exactly 20 years ago (Martinon et al., 2002), inflammasome research has today evolved into a spearhead of immunological research. The past two decades of inflammasome research have provided tremendous insight in the molecular mechanisms that regulate their activation, and uncovered critical roles in host defense against microbial pathogens and cellular perturbations. Much of our current knowledge of inflammasome biology has been gained from studies in murine model systems. In more recent times, significant progress has been made in translating this knowledge to the human system. These efforts have confirmed that inflammasome biology is well-conserved between rodent and human immune cells, but key differences have also been uncovered. For example, humans have a functional CARD8 inflammasome that is absent in rodents, and as discussed above, significant divergence between the human and murine NLRP1 inflammasome pathways have come to light. Recent studies have also revealed differential recognition of foreign and misplaced nucleic acids in the cytosol of murine macrophages and human monocytes. Furthermore, we have learned that distinct inflammasome sensors recognize the same bacterial PAMPs and toxins in human epithelial cells and myeloid cells, thus demonstrating a remarkable level of functional diversification in human inflammasome biology. The discovery that gain-of-function mutations in inflammasome receptor genes are responsible for the clinical manifestations of several auto-inflammatory diseases has also led to new therapeutic options for patients suffering from these 'inflammasomopathies'. Moreover, clinical trials with IL-1 blocking therapeutics - and the CANTOS study in particular - have provided unequivocal evidence that uncontrolled inflammasome activation plays a pathogenic role in a wide range of wide range of diseases that affect humanity. Furthermore, early in-human studies with selective NLRP3 inhibitors have recently been initiated, and several pharmacological tool compounds targeting GSDMD have been reported. However, despite these and many other groundbreaking developments, our understanding of human inflammasome biology remains fragmented. More emphasis should be placed on translating research findings from animal studies into the human system in the future. Novel state-of-the art research tools, such as CRISPR/CAS9 gene editing, organoid systems and human induced pluripotent stem cells, should be exploited more fully to investigate inflammasome pathways in disease-relevant experimental systems. It is clear that inflammasome research is as vibrant as it has ever been, and the recent progress described in this review strongly suggest that the next twenty years undoubtedly have another rollercoaster of new insights in store.

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DECLARATION OF INTERESTS

The authors declare that they have no conflict of interest.

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FIGURE LEGENDS

Figure 1: Schematic representation of the canonical and non-canonical inflammasomes. In the canonical inflammasome pathways, assembly of the inflammasome sensors together with the effector pro-CASP1, and in some cases with the adaptor protein ASC, leads to CASP1 activation. In the non-canonical pathway, detection of GBP-coated cytosolic LPS leads to CASP4/5 activation.

Figure 2: Schematic overview of the domain structures of the human inflammasome sensors, and reported stimuli of human inflammasome sensors and their respective murine counterparts.

Figure 3: Overview of the non-canonical and the canonical NLRP3 inflammasome and the crosstalk between these pathways. The NLRP3 inflammasome typically requires a priming and an activation signal for its assembly. Priming can be achieved by TLR4-stimulation with LPS and K^+ efflux is required for activation. The activated NLRP3 sensor, bound by NEK7, recruits ASC and pro-CASP1, followed by cleavage of pro-IL-1β, pro-IL-18 and GSDMD. Finally there is release of mature IL-1β and IL-18 and pyroptosis of the cell. In the non-canonical pathway, detection of cytosolic LPS leads to caspase-4/5 activation, GSDMD cleavage and pyroptosis. GSDMD pore formation can lead to a K^+ efflux as well, resulting in secondary NLRP3 inflammasome activation and associated cytokine secretion.

Figure 4: Schematic representation of the activation mechanisms of the NAIP/NLRC4, AIM2, Pyrin, NLRP1 and CARD8 inflammasome. A typical inducer of the NLRC4 inflammasome is needle protein of bacterial type III secretion systems, which is sensed by NAIP. With its CARD domain, NLRC4 can directly recruit pro-CASP1 for efficient inflammasome assembly. The HIN200 domain of AIM2 binds dsDNA and provokes recruitment of ASC and pro-CASP1. The Pyrin inflammasome is sensitive to bacterial toxins that inactivate RhoA GTPases, resulting in consolidation of the active inflammasome by binding with ASC and pro-CASP1. The NLRP1 and CARD8 inflammasome can get activated by VbP, which abolishes the inhibition of NLRP1 and CARD8 by dpp9, or by viral proteases. Proteasomal degradation of the N-terminal portion allows for inflammasome assembly of the C-terminal part. While NLRP1 interacts with pro-CASP1 through ASC recruitment, CARD8 is not able to recruit ASC but directly binds with pro-CASP1. The assembly of all of these inflammasomes induces the activation of CASP1 and results in GSDMD-mediated pore formation, mature cytokine release and pyroptosis.

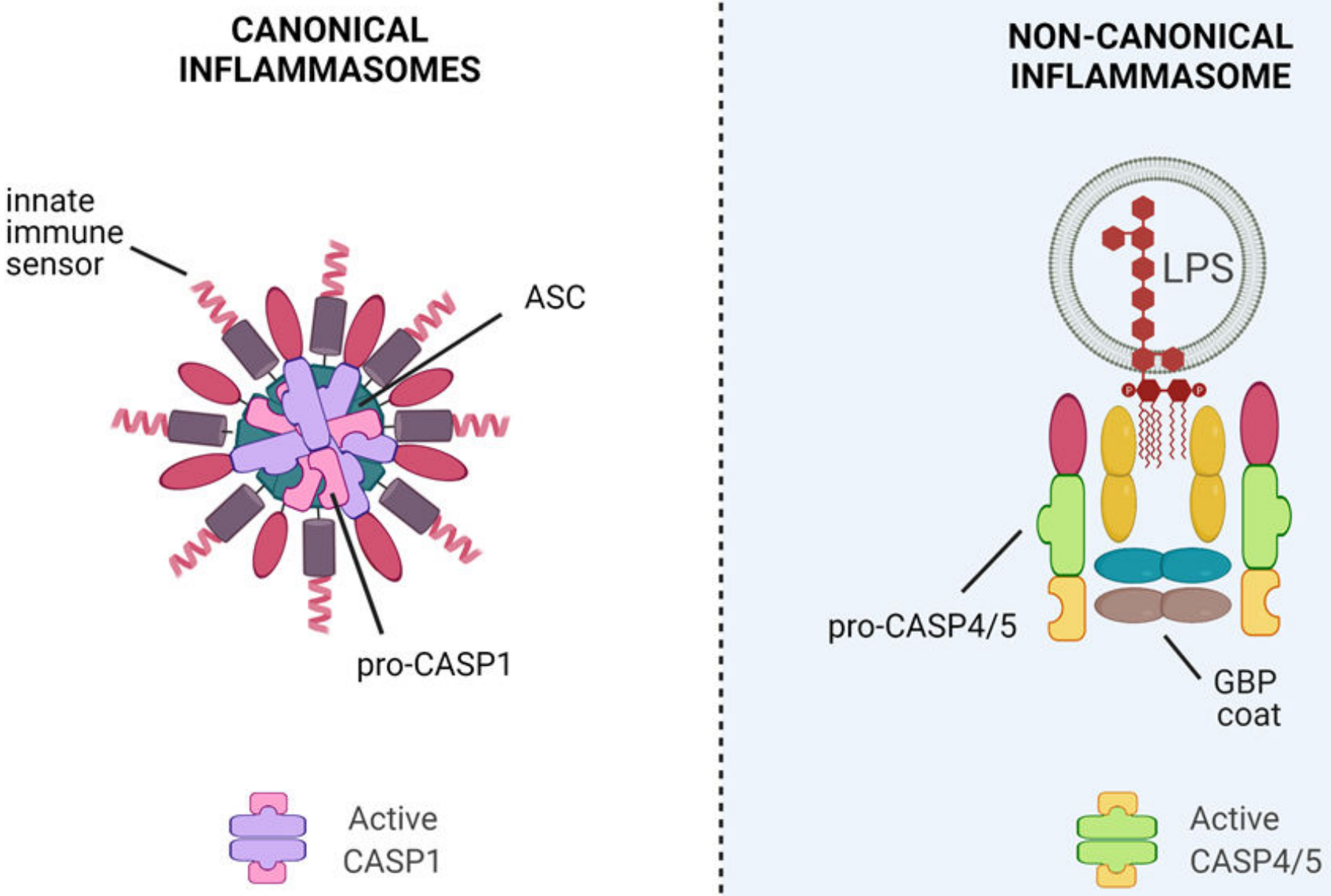

CANONICAL
INFLAMMASOMES
innate
immune
sensor
ASC
pro-CASP1
Active
CASP1
NON-CANONICAL
INFLAMMASOME
LPS
pro-CASP4/5
GBP
coat
Active
CASP4/5

Figure 1

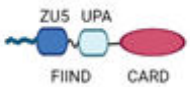
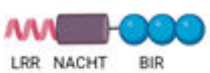
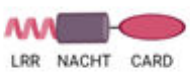
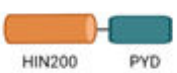
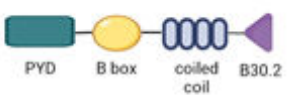
HUMAN INFLAMMASOME SENSORS		STIMULI HUMAN SENSOR	STIMULI MURINE SENSOR
NLRP1		DPP8/9 inhibitors dsRNA UVB Viral proteases	*B. anthracis* lethal toxin DPP8/9 inhibitors
CARD8		DPP8/9 inhibitors Viral proteases	Not applicable
NLRP3		LPS ATP Ionophores Pore-forming & RhoA-activating bacterial toxins Particulate agents dsDNA and dsRNA	ATP Ionophores Pore-forming & RhoA-activating bacterial toxins Particulate agents dsRNA
NAIP		Bacterial flagellin T3SS needle proteins T3SS rod proteins	Bacterial flagellin T3SS needle proteins T3SS rod proteins
NLRC4			
AIM2		Cytosolic dsDNA	Cytosolic dsDNA
Pyrin		RhoA-inhibiting microbial toxins	RhoA-inhibiting microbial toxins

Figure 2

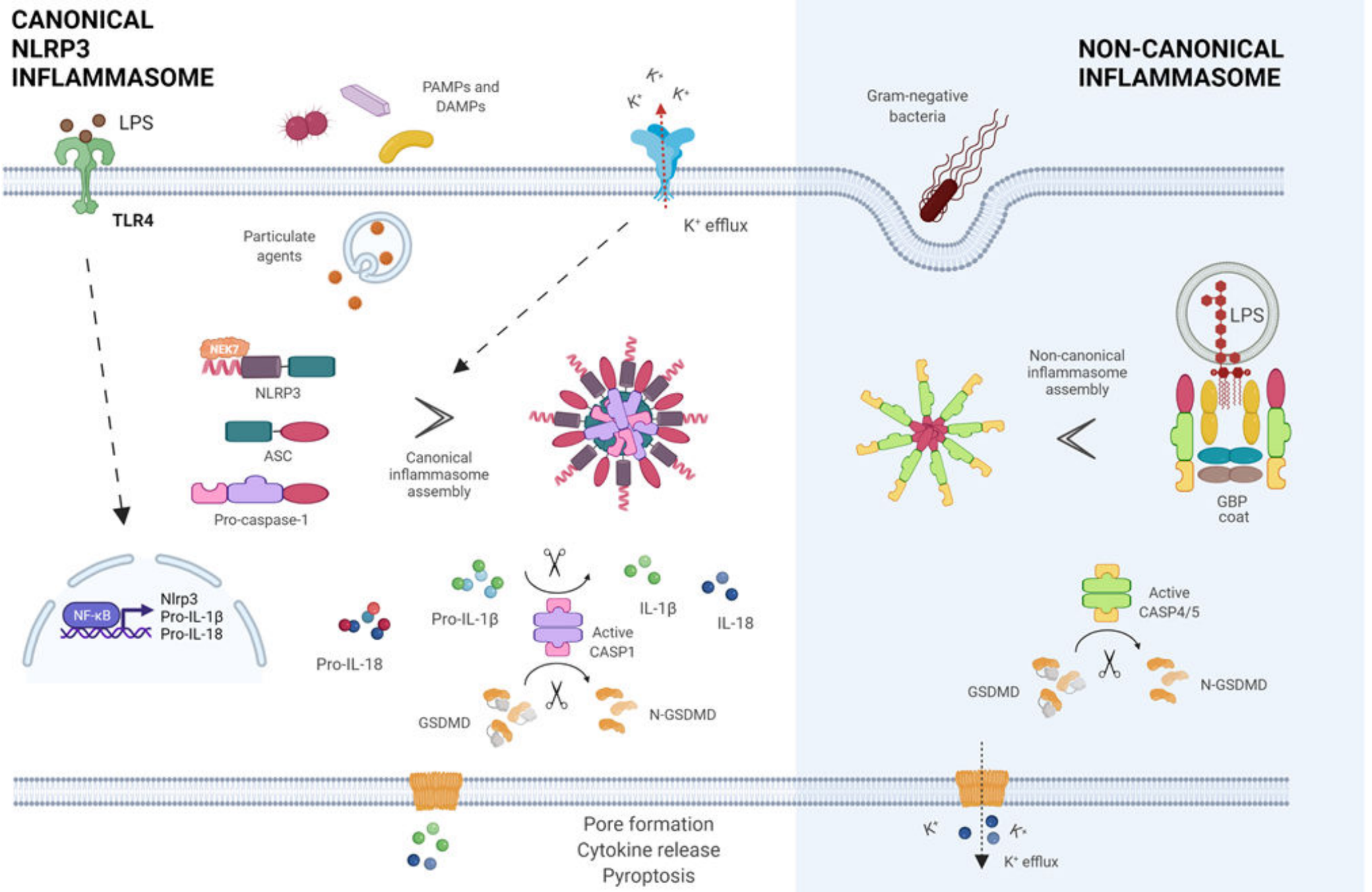

CANONICAL
NLRP3
INFLAMMASOME
NON-CANONICAL
INFLAMMASOME
LPS
TLR4
PAMPs and
DAMPs
K+
K+
K+
K+ efflux
Gram-negative
bacteria
Particulate
agents
NEK7
NLRP3
ASC
Pro-caspase-1
Canonical
inflammasome
assembly
Non-canonical
inflammasome
assembly
LPS
GBP
coat
NF-κB
Nlrp3
Pro-IL-1β
Pro-IL-18
Pro-IL-18
Pro-IL-1β
Active
CASP1
IL-1β
IL-18
GSDMD
N-GSDMD
Active
CASP4/5
GSDMD
N-GSDMD
Pore formation
Cytokine release
Pyroptosis
K+
K+
K+ efflux

Figure 3

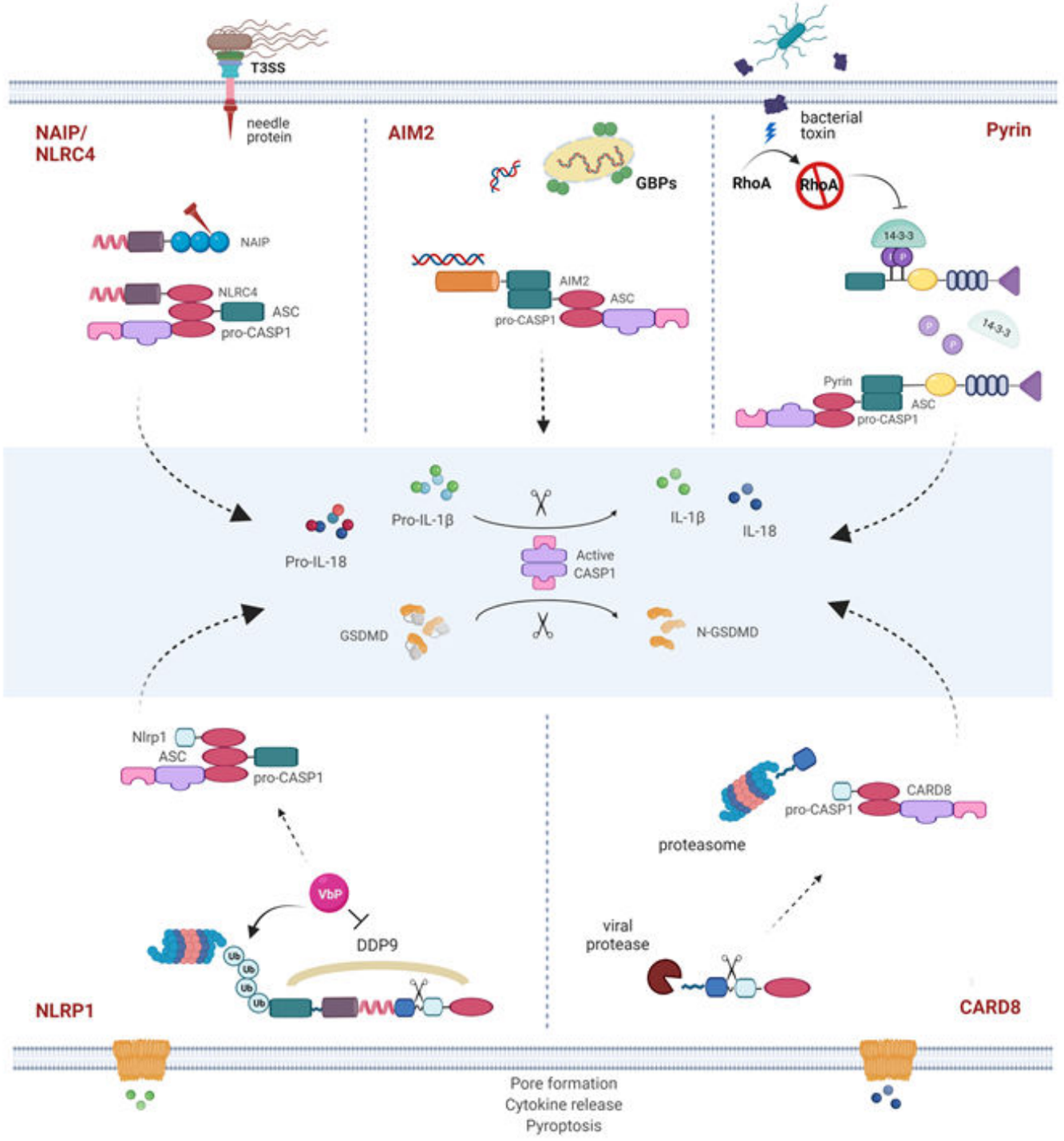

T3SS
needle protein
NAIP/ NLRC4
NAIP
NLRC4
ASC
pro-CASP1
AIM2
GBPs
AIM2
ASC
pro-CASP1
bacterial toxin
Pyrin
RhoA
RhoA
14-3-3
14-3-3
Pyrin
ASC
pro-CASP1
Pro-IL-18
Pro-IL-1β
Active CASP1
IL-1β
IL-18
GSDMD
N-GSDMD
Nlrp1
ASC
pro-CASP1
VbP
DDP9
NLRP1
proteasome
CARD8
pro-CASP1
viral protease
CARD8
Pore formation
Cytokine release
Pyroptosis

Figure 4